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From: STIC-Biotech/ChemLib
Sent: Thursday, October 09, 2003 6:01 AM
To: STIC-ILL
Subject: FW: In re: 10/005, 510 Journal article

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From: Ford, Vanessa
Sent: Wednesday, October 08, 2003 5:49 PM
To: STIC-Biotech/ChemLib
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SO VET.MED. (76, NO.8, 1185-86, 1981).*

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SO VETERINARY PARASITOLOGY, (MAY 1993) Vol. 47, No. 3-4, pp. 225-233.

SO Z PARASITENKD, (1983) 69 (1), 27-34.*

SO Revista Brasileira de Parasitologia Veterinaria, (1995) Vol. 4, No. 1, pp.*
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FEMSLE 05910

A simple and reliable method of producing in vitro infections of *Cryptosporidium parvum* (Apicomplexa)

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Abstract: A variety of techniques have been used to infect cell monolayers in culture with the protozoan, *Cryptosporidium parvum*. However, most of these methods rely on the use of trypsin and/or bile salts to excyst sporozoites in vitro, followed by washing sporozoites free of excystation solution prior to their addition to subconfluent monolayers. This method not only increases the amount of time required to establish infections in vitro, but also results in prolonged exposure of free sporozoites to environmental conditions. Here we report a simple, fast, and efficient method of obtaining consistent infections of *C. parvum* in cell monolayers. This technique relies on the ability of the parasite to excyst at 37°C but not at room temperature following pretreatment with sodium hypochlorite. By adding surface-sterilized oocysts directly to monolayers, sporozoites have access to host cells immediately upon excystation.

Key words: *Cryptosporidium parvum*; Coccidia; Cell culture

Introduction

Cryptosporidium parvum is a protozoan parasite capable of causing moderate to severe diarrheal illness in immunocompetent individuals and life-threatening disease in immunocompromised individuals [1,2]. Although over 20 papers have described various methods of infecting cell monolayers with this parasite [3], the lack of a good,

reproducible in vitro model system has impeded progress in studying parasite/host cell interactions. This report describes a simple, rapid, and reproducible method of obtaining viable, surface-sterilized oocysts and some of the parameters affecting development of these parasites in vitro.

Materials and Methods

Parasite preparation

Oocysts of *Cryptosporidium parvum* were passaged in 5-day-old neonate calves (*Bos taurus*), collected and stored in 2.5% (w/v) aqueous potassium dichromate ($K_2Cr_2O_7$) solution at 4°C, and finally purified on CsCl gradients as described previously [4–6]. Once pipetted from the

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gradients, the oocyst/CsCl suspension was mixed with ≥ 2 volumes ice-cold double-distilled water (ddH₂O) in 15-ml conical centrifuge tubes. Oocysts were pelleted by centrifugation at $1500 \times g$ for 20 min. The supernatant was removed and the oocysts transferred to 1.5 ml conical microfuge tubes and washed in ice-cold ddH₂O by centrifugation at $5000 \times g$ in a Costar^R model 10 microcentrifuge for 2 min. Once the supernatant had been removed, a 10% (v/v) ice-cold aqueous Clorox^R bleach solution was added. After vortexing, the suspension was allowed to sit on ice for 10 min. Oocysts were then pelleted by centrifugation for 2 min at $5000 \times g$. After the supernatant was removed, oocysts were washed 1 \times following resuspension in sterile, ice-cold PBS and again centrifuged at $5000 \times g$ for 2 min. The supernatant was again removed and oocysts resuspended in 1 ml cell culture media. A small aliquot of the oocyst suspension was diluted 1:100 in water or phosphate-buffered saline (PBS) and oocysts were quantitated using a haemocytometer. Oocysts were then diluted in cell culture medium to a final concentration of 5×10^5 oocysts ml⁻¹.

Excystation studies

The percentage of *C. parvum* oocysts that excysted was determined in all studies. This was accomplished by incubating aliquots of oocysts in cell culture medium at 37°C for 2 h, then scoring at least 100 oocysts as either empty or intact (unexcysted). After oocysts had aged beyond 6 weeks, which is normally the maximum length of time they are used to infect cell cultures in our laboratory, excystation studies were continued up to 90 days to correlate excystation of CsCl gradient-purified oocysts with age. In a separate experiment, we incubated individual aliquots of oocysts at both 37°C and 24°C and determined excystation at periodic intervals through 190 min. In other experiments where percent excystation was correlated with infection of cell monolayers, reduced excystation was artificially generated by prolonging surface sterilization of oocysts in 10% Clorox^R solution (above) for 60 min, and also performing the sterilizations at room temperature rather than on ice.

Cell culture

Madin-Darby bovine kidney cells (ATCC 6071) were maintained in 75-cm² tissue culture flasks. Cells were tested monthly for contamination with *Mycoplasma* spp. using 4',6-diamidino-2-phenylindole (DAPI) stain [7-9]. To lift cells from the surface of flasks, a 0.25% (w/v) trypsin/0.53 mM ethylenediamine tetraacetic acid (EDTA) in phosphate-buffered saline (PBS) solution was used. The cell culture medium consisted of RPMI 1640 with L-glutamine, supplemented with sodium bicarbonate, 2.2 g l⁻¹; HEPES buffer, 15 mM; penicillin, 100 U ml⁻¹; streptomycin, 100 μ g ml⁻¹; and amphotericin B, 0.25 μ g ml⁻¹. For routine cell passage, 5% fetal bovine serum (FBS) was used (maintenance medium) whereas 10% FBS was used whenever parasites were employed (growth medium).

14 h prior to inoculation with *C. parvum*, cells were plated onto 22-mm² glass coverslips in 6-well cluster plates at a concentration of 7×10^5 viable cells in a total volume of 3 ml. Cell viability was assessed using trypan blue exclusion (0.02% (w/v) in PBS) and numbers were quantitated using a haemocytometer. Plates were incubated at 37°C in a 5% CO₂/95% air humidified incubator.

Prior to infection of monolayers with *C. parvum*, the cell culture maintenance medium

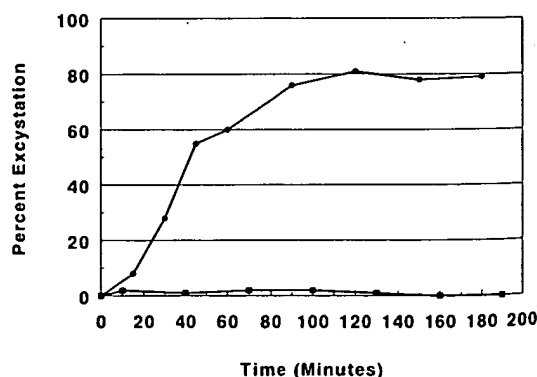


Fig. 1. Percent excystation (No. empty oocysts/No. empty + intact oocysts) of CsCl-purified and surface-sterilized oocysts of *Cryptosporidium parvum* at 37°C (circles) and room temperature (24°C; squares) over time. Oocysts were excysted in RPMI 1640 cell culture medium supplemented with 10% fetal bovine serum, 15 mM HEPES buffer, 2.2 g l⁻¹ sodium bicarbonate, and antibiotics.

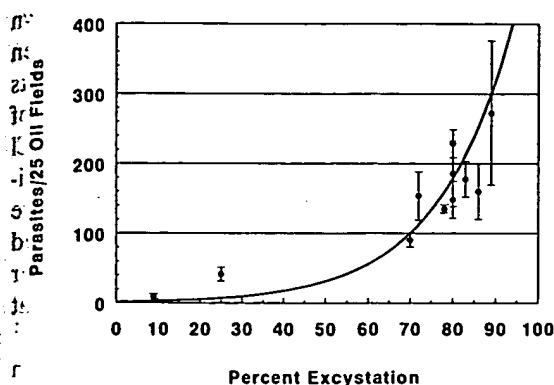


Fig. 2. Nomarski interference contrast photomicrographs of *Cryptosporidium parvum*. (A) CsCl-purified, surface-sterilized oocysts prior to addition to cell monolayer. Magnification $\times 620$. (B) Oocysts from (A), 60 min after elevating temperature to 37°C . Magnification $\times 620$. (C) Developmental stages of *Cryptosporidium parvum* in MDBK cells following the addition of surface-sterilized oocysts 68 h previously. Magnification $\times 1400$. Incubation was for 68 h in candle jars at 37°C in RPMI 1640 medium supplemented with 10% fetal bovine serum, 15 mM HEPES buffer, 2.2 g l^{-1} sodium bicarbonate, and antibiotics. Abbreviations: Ma, macrogamete; Me, meront; U, undifferentiated stage.

was removed and 2 ml oocyst suspension (1.25×10^6 oocysts ml^{-1}) in growth medium were added. Cluster plates were then placed into candle jars

as described previously [3,10,11] and incubated at 37°C . In order to remove most unexcysted oocysts, oocyst walls, and other toxic materials that may have been liberated from oocysts [3], plates were removed from candle jars at 2 h, monolayers washed $1 \times$ with PBS, and new growth medium was added. Cluster plates were then resealed into candle jars, the candles relit, and monolayers incubated at 37°C .

Parasite growth in vitro was assessed 68 h following the initial parasite inoculation by removing cluster plates from candle jars and viewing parasite infected monolayers under Nomarski interference contrast optics. A $10 \times$ ocular lens and $100 \times$ objective oil immersion lens were employed. Twenty-five oil fields were randomly examined per coverslip and total numbers of parasite developmental stages assessed, as has been described previously [11]. Each experiment involving parasite development in cell culture was performed in replicates of 4–6.

Results

Using the above protocol, oocysts of *C. parvum* could be surface-sterilized and in cell culture in

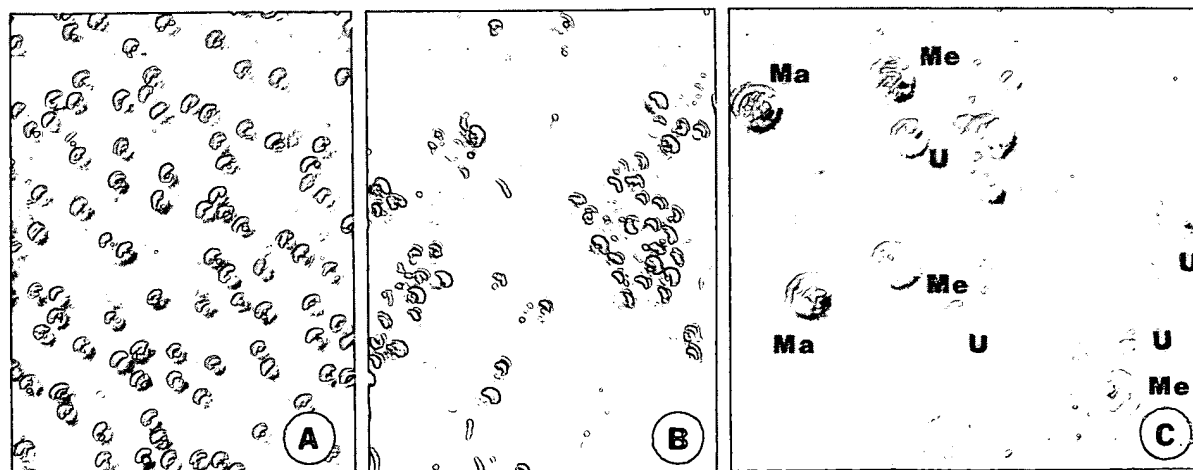


Fig. 3. Effects of percent excystation on numbers of parasite developmental stages in MDBK cells in vitro. Incubation was for 68 h in candle jars at 37°C in RPMI 1640 medium supplemented with 10% fetal bovine serum, 15 mM HEPES buffer, 2.2 g l^{-1} sodium bicarbonate, and antibiotics. The lower two data points were artificially generated by prolonged incubation of oocysts for 60 min at room temperature (24°C). The slope of the line was generated by exponential regression analysis.

about 1 h. These oocysts excysted rapidly and sigmoidally when the temperature was elevated to 37°C (Figs. 1, 2), and were clean of most debris (Fig. 2); excystation did not occur at room temperature (24°C). Excystation varied from 80–90% for oocysts processed within 2 weeks after being collected from calves, whereas excystation diminished to 50–68% when oocysts 60–90 days were used (data not shown). Percent excystation was correlated with numbers of parasites developing in vitro (Figs. 2, 3).

Discussion

We describe a rapid and simple protocol that we routinely employ to infect cell monolayers with *C. parvum*. We have used oocysts directly to infect cells previously [3,11], but have recently reduced the amount of time needed to manipulate the parasites. This method relies on previous observations that have shown *C. parvum* oocysts will readily excyst when the temperature is elevated to 37°C following treatment with 10% bleach [11,12]. Percent excystation of sodium hypochlorite-treated oocysts are nearly identical to that observed when bile salts are used [13], and all that is apparently needed for excystation is a reagent that affects the oocyst wall and suture [14]; non-pretreated oocysts excyst poorly [15,16].

The use of intact oocysts to infect cell cultures is superior over excysted sporozoites for two reasons. First, cell monolayers can be infected quickly and reproducibly, without the need to purify sporozoites by anion exchange chromatography [12], Percoll or CsCl gradients [5,17]. Second, sporozoites have almost immediate access to host cells and spend a minimal amount of time exposed to the extracellular environment. Sporozoites liberated from oocysts are frail and known to lyse easily [18] and, thus, viability is always uncertain whenever sporozoites are manipulated outside of the oocyst.

Our results suggest excystation levels below about 70% are not useful for in vitro assays when intact oocysts are used as inoculum. Percent excystation appears to fall to 70% when oocysts are about 2 months of age. These results are consis-

tent with the finding of others who have shown that oocyst viability begins to decrease between the second and third month [4,5]. Our results undoubtedly overestimate percent excystation of oocysts due to long-term storage as the CsCl gradient is thought to select against morphologically aberrant and inviable oocysts [5]. Indeed, we always note that numbers of oocysts harvested from CsCl gradients drop off substantially after oocysts are stored for ≥ 2 months in $K_2Cr_2O_7$ at 4°C.

Examining infected cell cultures at about 68 h is important because a significant number of meronts and gametes are present. Prolonged incubations sometimes result in slightly higher numbers of developmental stages and, occasionally, oocysts. However, monolayers often begin to slough after 68 h, and it is sometimes difficult to distinguish newly formed oocysts from the occasional residual oocyst from the original inoculum.

Acknowledgements

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References

- 1 Current, W.L. and Garcia, L.S. (1991) Cryptosporidiosis. Clin. Microbiol. Rev. 4, 325–358.
- 2 Ungar, B.L.P. (1990) Cryptosporidiosis in humans (*Homo sapiens*). In: Cryptosporidiosis of Man and Animals (Dubey, J.P., Speer, C.A. and Fayer, R., Eds.), pp. 59–82. CRC Press, Boca Raton, FL.
- 3 Eggleston, M.T., Tilley, M. and Upton, S.J. (1994) Enhanced development of *Cryptosporidium parvum* in vitro by removal of oocyst toxins from infected cell monolayers. Proc. Helminthol. Soc. Wash. 61, 118–121.
- 4 Current, W.L. (1990) Techniques and laboratory maintenance of *Cryptosporidium*. In: Cryptosporidiosis of Man and Animals (Dubey, J.P., Speer, C.A. and Fayer, R., Eds.), pp. 31–58. CRC Press, Boca Raton, FL.
- 5 Taghi-Kilani, R. and Sekla, L. (1987) Purification of *Cryptosporidium parvum* oocysts and sporozoites by cesium chloride and percoll gradients. Am. J. Trop. Med. Hyg. 36, 505–508.
- 6 Tilley, M. and Upton, S.J. (1990) Electrophoretic characterization of *Cryptosporidium parvum* (KSU-1 isolate)

- (Apicomplexa: Cryptosporidiidae). Can. J. Zool. 68, 1513-1519.
- 7 Mitchell, A. and Finch, L.R. (1977) Pathways of nucleotide biosynthesis in *Mycoplasma mycoides* subsp. *mycoides*. J. Bacteriol. 130, 1047-1054.
 - 8 Russell, W.C., Newman, C. and Williamson, D.H. (1975) A simple cytochemical technique for demonstrating DNA in cells infected with mycoplasmas and viruses. Nature 253, 461-462.
 - 9 Uphoff, C.C., Gignac, S.M. and Drexler, H.G. (1992) *Mycoplasma* contamination in human leukemia cell lines. J. Immunol. Methods 149, 43-53.
 - 10 Tilley, M. and Upton, S.J. (1988) A comparative study of the development of *Eimeria nieschulzi* in vitro under aerobic and reducing conditions. J. Parasitol. 74, 1042-1045.
 - 11 Upton, S.J., Tilley, M., Mitschler, R.R. and Oppert, B.S. (1991) Incorporation of exogenous uracil by *Cryptosporidium parvum* in vitro. J. Clin. Microbiol. 29, 1062-1065.
 - 12 Riggs, M.W. and Perryman, L.E. (1987) Infectivity and neutralization of *Cryptosporidium parvum* sporozoites. Infect. Immun. 55, 2081-2087.
 - 13 Woodmansee, D.B. (1987) Studies of in vitro excystation of *Cryptosporidium parvum* from calves. J. Protozool. 34, 398-402.
 - 14 Reduker, D.W., Speer, C.A. and Blixt, J.A. (1985) Ultrastructural changes in the oocyst wall during excystation of *Cryptosporidium parvum* (Apicomplexa; Eucoccidiorida). Can. J. Zool. 63, 1892-1896.
 - 15 Fayer, R. and Leek, R.G. (1984) The effects of reducing conditions, medium, pH, temperature, and time on in vitro excystation of *Cryptosporidium*. J. Protozool. 31, 567-569.
 - 16 Reduker, D.W. and Speer, C.A. (1985) Factors influencing excystation of *Cryptosporidium* oocysts from cattle. J. Parasitol. 71, 112-115.
 - 17 Arrowood, M.J. and Sterling, C.R. (1987) Isolation of *Cryptosporidium* oocysts and sporozoites using discontinuous sucrose and isopycnic Percoll gradients. J. Parasitol. 73, 314-319.
 - 18 Robertson, L.J., Campbell, A.T. and Smith, H.V. (1993) In vitro excystation of *Cryptosporidium parvum*. Parasitology 106, 13-19.